



Faculty of Resource Science and Technology

Isolation and Detection of *Burkholderia* species in Soil Sample

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Isolation and Detection of *Burkholderia* species in Soil Sample

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**This project is submitted in partial fulfillment of the requirements for the
Degree of Bachelor of Science with Honours (Resource Biotechnology)**

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Declaration

I hereby declare that this thesis entitled “Isolation and Detection of *Burkholderia* species in Soil Sample” is based on my original works except quotations and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at Universiti Malaysia Sarawak or other institutions.

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Table of contents

Acknowledgements	I
Declaration	II
Table of contents	III
List of abbreviations	V
List of tables	VI
List of figures	VII
Abstract	1
1.0 Introduction	2
2.0 Literature review	4
2.1 <i>Burkholderia pseudomallei</i>	4
2.2 Melioidosis	6
2.3 Previous studies on <i>B. pseudomallei</i> in soil and the environment	7
3.0 Materials and Methods	9
3.1 Materials	9
3.1.1 Soil sampling	9
3.1.2 Ashdown's selective broth	9
3.1.3 Ashdown's selective agar	9
3.1.4 Chromosomal DNA extraction	10
3.1.5 PCR analysis	10
3.1.6 Gel electrophoresis	10
3.2 Methods	11
3.2.1 Soil sampling	11
3.2.2 Ashdown's selective broth preparation	13
3.2.3 Ashdown's selective agar preparation	13

3.2.4	Soil sample culture	13
3.2.5	Chromosomal DNA extraction	14
3.2.6	PCR analysis and Gel electrophoresis	15
4.0	Results and Discussion	16
4.1	Sampling location parameter	16
4.2	Sampling site	17
4.3	Morphology observation	18
4.4	PCR analysis	21
5.0	Conclusion	26
6.0	References	27
7.0	Appendices	29
7.1	Agarose gel assay	29
7.2	Study form	32

List of Abbreviations

GPS	global positioning system
ng	nano gram
PCR	polymerase chain reaction
spp.	species
UHQ	ultra high quality
UV	ultra violet
μ	micro
w/v	weight per volume

List of tables

Tables	Caption	Page
Table 3.2.1	List of targeted sites and random sites	12
Table 3.2.6	Bur 3/4 PCR condition	15
Table 4.1	Location parameter list	16
Table 4.2	List of targeted area and random area with their respective site number	18
Table 4.3.1	The number of subculture colony and morphology of pure colony per sample	19
Table 4.3.2	Cultures morphology for plate B	21
Table 4.4	Percentage of positive Bur 3/4 PCR for random area and targeted area	23
Table 7.2.1	Study form for Riverbank, Ulu Lubai	32
Table 7.2.2	Study form for buffer zone, Ulu Lubai	33
Table 7.2.3	Study form for pineapple orchard	34
Table 7.2.4	Study form for open forest, Ulu Lubai	35
Table 7.2.5	Study form for Hill	36
Table 7.2.6	Study form for residence area	37
Table 7.2.7	Study form for UNIMAS- student walkway	38
Table 7.2.8	Study form for Kpg. Sebayor	39

List of figures

Figures	Caption	Page
Figure 2.1	<i>Burkholderia</i> spp. in Ashdown's agar	5
Figure 3.2.1	Sampling site location at Ulu Lubai, Limbang, Sarawak	11
Figure 3.2.4	Bacterial culture method	14
Figure 4.3	The morphology of the subculture in Ashdown's selective agar	20
Figure 4.4.1	Agarose gel staining with SYBR® Safe dye for Bur 3/4 PCR	21
Figure 4.4.2	TTSI real-time PCR analysis	24
Figure 7.1.1	Chelex number 1-8	29
Figure 7.1.2	Chelex number 9-24	29
Figure 7.1.3	Chelex number 25-40	29
Figure 7.1.4	Chelex number 41-44	29
Figure 7.1.5	Chelex number 45-60	30
Figure 7.1.6	Chelex number 61-77	30
Figure 7.1.7	Chelex number 78-103	30
Figure 7.1.8	Chelex number 104-110	30
Figure 7.1.9	Chelex number 111-128	31
Figure 7.1.10	Chelex number 129-146	31
Figure 7.1.11	Chelex number 147 & 148	31

ABSTRACT

Melioidosis may be fatal to the infected human and the animal. Exposure to the soil with high content of *Burkholderia pseudomallei* may result in fatal infections such as melioidosis. These bacteria dominating a wide range of ecological niches and mainly found in the soil. The prevalence of *Burkholderia* spp. in soil is high in disturbed areas such paddy field, high water contain soil and the presence of animals. Therefore, in this study, we isolate the *Burkholderia* spp. from soil sampled at different areas that probably have high content of these bacteria. The soils samples then were subjected to bacterial culture using Ashdown's broth before further analysis with PCR of the Bur 3/4 gene and type III secretion system gene. Analysis showed 58% were positive for *Burkholderia* spp. while 2% of the isolates were tested weak positive for *B. pseudomallei*.

Key words: Ashdown's broth, *B. pseudomallei*, melioidosis, soil, Bur 3/4 gene, environmental niches.

ABSTRAK

Melioidosis boleh menyebabkan maut kepada manusia dan haiwan yang dijangkiti. Pendedahan kepada tanah yang tinggi kandungan *Burkholderia pseudomallei* boleh menyebabkan jangkitan yang boleh membawa maut seperti Melioidosis. Bakteria ini mendominasi pelbagai ekologi alam khususnya di dalam tanah. Lazimnya, species *Burkholderia* terdapat di dalam kawasan yang telah diterokai dan sawah padi. Kandungan air yang tinggi di dalam tanah dan kehadiran haiwan di sesuatu tempat juga adalah salah satu faktor menyebabkan kehadiran bakteria ini. Oleh sebab itu, dalam kajian ini, kami mengasingkan spesies *Burkholderia* dari tanah sampel di kawasan yang berbeza yang mungkin mempunyai tinggi kandungan bakteria ini. Sampel tanah kemudian dikultur menggunakan cecair media Ashdown sebelum di analisis dengan PCR daripada Bur 3/4 gen dan jenis III sistem rembesan gen. Analisis menunjukkan 58% daripada sampel coloni positif spesies *Burkholderia* dan 2% daripadanya mengandungi gen *B. pseudomallei*.

Kata kunci: Cecair media Ashdown, *B. pseudomallei*, melioidosis, tanah, Bur 3/4 gene, ekologi alam.

1.0 Introduction

Burkholderia genus is a large group of bacteria comprised of more than 70 species which have been dominating a wide range of ecological niches including the environment and human respiratory tract (Coenye & Vandamme, 2003; Angus *et al.*, 2014). The *Burkholderia* genus previously part of *Pseudomonas*, refers to a group of virtually ubiquitous Gram-negative, motile, obligatory aerobic rod-shaped bacteria including both animal and plant pathogens, as well as some environmentally important species (Estrada-de los Santos *et al.*, 2013).

According to Coenye and Vandamme (2003), these bacteria are normally exploited for plant growth stimulation, bio-remediation and bio-control purposes. However, the different agricultural activities - crop cycle, nonstop cropping and spadework, and the presence of animals such as livestock, dogs, wallabies as well as the soil texture (red brown clay) contribute to higher presence of *Burkholderia pseudomallei* (*B. pseudomallei*) which have been shown to cause variations of microbial emergence in soil (Salles, Veen & Elsas, 2004; Kaestli *et al.*, 2009).

The excessive concentration of *Burkholderia species* (spp.) in our surrounding causes infections in humans such as opportunistic infections in cystic fibrosis (CF) patients (Mahenthiralingam *et al.*, 2005) and melioidosis (Chen *et al.*, 2010). In this project, we focus on *B. pseudomallei*, the causative agent of melioidosis which cause fatal to the infected human and the cases even found in animal (Brook *et al.*, 1997; Limmathurotsakul *et al.*, 2013).

B. pseudomallei is a sub-species of the *Burkholderia* family which is a free-living organism of water and soil (Brook *et al.*, 1997). These bacteria are endemic in Southeast Asia and northern Australia where melioidosis is directly linked to increased rainfall and extreme weather events (Baker *et al.*, 2011). According to Wuthiekanun *et al.* (2005) and Chen *et al.*

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(2010), melioidosis patients are usually recreational or occupational with exposure to mud and surface water, particularly rice farmers. The disease can be occur after bacterial contamination of the skin through contact with soil and can also cause pneumonia from inhalation of aerosidised soil particles (Hassan *et al.*, 2010; Chen *et al.*, 2010).

In Taiwan, up to year 2000, small cases of melioidosis were recorded and it was categorized as being acquired during travels to the endemics region and the pathogen did not have been isolated from the environment (Chen *et al.*, 2010). However, the number of melioidosis rapidly increased after a typhoon followed by flood at Taiwan in 2005 (Chen *et al.*, 2010). This suggest that the prevalence of *B. pseudomallei* in the environment may increase in the event of extreme weathers as reported in Taiwan (Chen *et al.*, 2010).

Therefore, following the case in Taiwan and recent flood events in Sarawak during January 2015, the wide utilization of lands and forest such as reforestation, residential area development and farming, the isolation of *Burkholderia* spp. from soil is of interest. The objectives of this study are

1. To isolate and identify *Burkholderia* spp. from soil samples
2. To identify the factors that influence the ecology of *B. pseudomallei* in the soil

The soil was sampled from target and random sites. The soil samples was cultured with Ashdown's broth before spread onto the Ashdown's agar to get the pure culture (Kaestli *et al.*, 2009). The pure culture then undergo polymerase chain reaction with Bur 3/4 gene to indicate the presence of the *Burkholderia* spp. in the soil sample (Payne *et al.*, 2005).

2.0 Literature review

2.1 *Burkholderia pseudomallei*

Burkholderia pseudomallei also known as *Pseudomonas pseudomallei*, is a Gram-negative bacteria, aerobic, motile rod shape bacterium and bipolar which can infect human and animals (Payne *et al.*, 2005; Pringle, 2010 & Mahbub *et al.*, 2015). *B. pseudomallei* is endemic to Southeast Asia, northern Australia and temperate regions that border the equator, and it is the etiological agent of melioidosis in humans and animals (Coenye & Vandamme, 2003, Kaestli *et al.*, 2009, Chen *et al.*, 2010 & Lau *et al.*, 2014). According to Lau *et al.* (2014), although melioidosis is mainly endemic in Southeast Asia and northern Australia, it also has increasingly being reported in countries outside the Asia-Pacific region including India, Mauritius, United States and Africa.

B. pseudomallei is a natural saprophyte that can be isolated from soil, groundwater, stagnant streams, rice paddies and ponds, and in major natural reservoirs of the bacteria (Baker *et al.*, 2011 & Lau *et al.*, 2014). *B. pseudomallei* can infect humans and animals through skin abrasion contact with contaminated soil, ingestion and inhalation of aerosol (Coenye & Vandamme, 2003, Pringle, 2010 & Lau *et al.*, 2014). Besides, *B. pseudomallei* is a resilient organism that is capable of surviving in hostile environmental conditions including prolonged nutrient deficiency antiseptic and detergent solutions, acidic environments, a wide temperature range and dehydration but not exposure to UV light (Cheng & Currie, 2005).

According to Lau *et al.* (2014), the diagnosis of melioidosis can be difficult because its incubation period varies widely from 2 days to 26 years as the bacterium may not be readily isolated from clinical specimens. *B. pseudomallei* cultures also can be misidentified between *B.*

pseudomallei and other closely related species such as *B. thailandensis*, *B. cepacia* complex, *Pseudomonas* spp., *Burkholderia vietnamiensis*, *Stenotropomonas maltophilia* and *Chromobacterium violaceum* which can cause an infection that similar to melioidosis symptoms (Peacock *et al.*, 2005 & Novak *et al.*, 2006). In some regions, *B. pseudomallei* is being classified to a category B select agent by the Centers for Disease Control and Prevention (CDC) because of its high infectivity, severity of disease and environmental persistence (Novak *et al.*, 2006).

The possible identification of colonies of *B. pseudomallei* can be made from their characteristic purple color and dry and wrinkled appearance after 24 h to 48 h of incubation on Ashdown's selective agar (Peacock *et al.*, 2005 & Chantratita *et al.*, 2007). Figure 2.1 show the example of the *Burkholderia* spp. isolated from sampling site in Ashdown's agar.



Figure 2.1: *Burkholderia* spp. in Ashdown's agar. The example of *Burkholderia* spp. isolate from sampling site.

These misidentifications were caused by some biochemical identification methods which may be inaccurate and *B. pseudomallei* also undergoes adaptation which altered surface expression and colony morphology that facilitates bacterial survival in vivo (Novak *et al.*, 2006 & Chantratita *et al.*, 2007). Hence, molecular-based identification methods are much more accurate and specific. For instance, Novak and co-workers showed that the real-time PCR assay which targets the open reading frame 2 (*orf2*) of the *B. pseudomallei* type III secretion system (TTS1) exactly distinguished *B. pseudomallei* from non-*B. pseudomallei* (2006). In addition, Payne *et al.* (2005) also described a novel PCR assay targeting the *recA* gene of *Burkholderia* which is able to be sequenced to differentiate both putative and known *Burkholderia* spp. and Bcc nucleotide by phylogenetic analysis.

2.2 Melioidosis

Melioidosis is more common among the immunocompromised and is an increasing public health concern in endemic regions (Brook *et al.*, 1997). Melioidosis is the third most common cause of death due to infectious disease after HIV/AIDS and tuberculosis as reported in northeast Thailand (Limmathurotsakul *et al.*, 2013) and it is an endemic disease in tropical Australia (Kaestli *et al.*, 2009) where it has been acknowledged as the common cause of fatal community acquired bacteremic pneumonia (Limmathurotsakul *et al.*, 2013).

Cheng and Currie stated that Alfred Whitmore and his assistant C. S. Krishnaswami in Rangoon, Burma, in 1911 described melioidosis as a “glanders-like” disease among morphia addicts where they recognized a new organism that fulfilled Koch’s postulates for causation of

disease (2005). In 1932, Stanton and Fletcher named the melioidosis from the Greek “melis”- distemper of asses and “eidos”- resemble (Cheng & Currie, 2005).

Melioidosis has associated co-morbidities such as diabetes, renal disease and alcoholism, causing infections that can resemble typhoid, tuberculosis and malaria (Kaestli *et al.*, 2010). According to Hassan *et al.* (2010), patient with diabetes mellitus constituted the major underlying risk factor for developing and dying from melioidosis. Patients with diabetes also suffered significantly more mortality from melioidosis compared to those who did not have this risk factor (Hassan *et al.*, 2010).

2.3 Previous study on *B. pseudomallei* in soil and environment studies

A study by Kaestli *et al.* (2009) in the Northern Territory of Australia reported that *B. pseudomallei* has been detected in untreated water supply and connection with monsoonal rain or extreme weather event. In another study, Castle Hill in Townsville, Queensland, has been recognized as a potential reservoir for melioidosis which is directly linked to heavy rainfall and extreme weather events (Baker *et al.*, 2011). Baker *et al.* found that lower part of Castle Hill contained high number of *B. pseudomallei* especially after heavy rainfall in wet season (2011). This is because the water flows from high places at Castle Hill sweep along these bacteria and causes the accumulation *B. pseudomallei* at the lower part of the hill (Baker *et al.*, 2011).

Agricultural work activities have also been shown to expose farmer to high level of *B. pseudomallei* in Thailand (Chen *et al.*, 2010 & Kaestli *et al.*, 2009). In addition, Kaestli *et al.* (2009) reported that *B. pseudomallei* can also found during dry seasons in undisturbed and environmentally disturbed sites such as farms and residential areas.

In Malaysia, the cases were reported from patient in Johor, Pahang state and Kuala Lumpur, but the cases pattern in Kedah were unknown despite its proximity and as the largest rice growing region of the country similar to Thailand (Hassan *et al.*, 2010). Besides that, *B. pseudomallei* also has been isolated from hospitals in the central region of Sarawak such as Bintulu, Kapit and Sibul (Podin *et al.*, 2014).

3.0 Materials & method

3.1 Materials

3.1.1 Soil sampling

Local auger 'sandak'

Hand trowel

Zip-lock bags

Global positioning system (GPS) unit (GPSMAP®G2S, Garmin, Kansas, USA)

70% ethanol

Light proof bags/box

3.1.2 Ashdown's selective broth

Tryptone (Oxoid, Thermo Fisher Scientific Co., MA, USA)

0.1% Crystal violet (Sigma, St. Louis, MO, USA)

UHQ water

Colistin (Sigma, St. Louis, MO, USA)

3.1.3 Ashdown's selective agar

Tryptone (Oxoid, Thermo Fisher Scientific Co., MA, USA)

Glycerol

1% aqueous neutral red (Sigma, St. Louis, MO, USA)

0.1% crystal violet (Sigma, St. Louis, MO, USA)

Agar powder (Sigma, St. Louis, MO, USA)

UHQ water

Gentamicin (Sigma, St. Louis, MO, USA)

3.1.4 Chromosomal DNA extraction

Centrifuge (Eppendorf, Hamburg, Germany)

10% Chelex 100 (Bio-Rad Laboratories, CA, USA)

3.1.5 PCR analysis

HotStar *Taq* DNA polymerase (Qiagen, Dusseldorf, Germany)

250 μ M of deoxynucleoside triphosphate (Fermentas, Thermo Scientific, PA, USA)

1x PCR buffer (including 1.5 mM MgCl₂) (Qiagen, Dusseldorf, Germany)

10 pmol of each appropriate oligonucleotide primer

10-50 ng of template DNA

3.1.6 Gel electrophoresis

1x TBE buffer Agarose powder

SYBR[®] Safe DNA gel stain (Invitrogen[™], USA)

6x Loading Dye DNA Ruler

Bio-Rad ChemiDoc[™] XRS Gel Documentation system (Bio-Rad Laboratories, USA)

Safe Imager[™] Blue-Light Transilluminator

3.2 Methods

3.2.1 Soil sampling

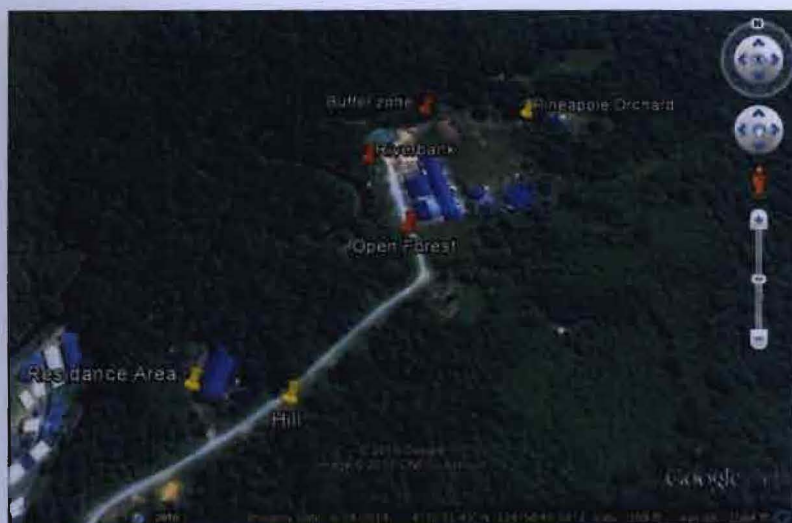


Figure 3.2.1: Sampling site location at Ulu Lubai, Limbang, Sarawak. The red pin indicates the random sites and yellow pins are targeted sites.

The samples were taken from nine sites, 5 targeted sites and 4 random sites. The samples were sampled at UNIMAS and Ulu Lubai, Limbang. The samples at Ulu Lubai, as shown in the Figure 3.2.1 was sampled after flooded in January 2015 except at riverbank (before flood). The Table 3.2.1 below shows the list of targeted sites and random sites. A targeted area was described using the following parameters which indicate the probability of higher presence of *Burkholderia* spp. in soil:

- a) Waterlogged and heavy clay soils (Inglis *et al.*, 2006)
- b) High water containing soil - muddy, moist, clay-rich soil and pooled surface water (Kaestli *et al.*, 2009)

- c) Recent soil disturbances such as excavation and plowing (Cheng & Currie, 2005; Kaestli *et al.*, 2009)
- d) Presence of animals (Kaestli *et al.*, 2009)

Table 3.2.1: List of targeted sites and random sites

Targeted site	Random site
UNIMAS sewage lake	Riverbank, Ulu Lubai
Pineapple orchard, Ulu Lubai	Buffer zone, Ulu Lubai
Hill, Ulu Lubai	Open forest, Ulu Lubai
Residence area, Ulu Lubai	UNIMAS (Student walkway; Cempaka college – FEB)
Kpg. Sebayor	

Upon arriving at the site, five holes were dug and the depth (15cm-30cm) was depends on the soil texture by using auger called ‘sandak’. The only exception is the sampling site at UNIMAS where 7 holes were dug. A hand trowel was been used to transfer soil into labeled zip-lock bags. The bags of soil were protected from direct sunlight as ultraviolet (UV) light had been reported to kill *B. pseudomallei* and *Burkholderia* spp.

The landscape such as water run-off, water logging, distance to stream, type of vegetation and elevation, was recorded. The point location of each hole also was recorded using a handheld global positioning system (GPS) unit (GPSMAP®G2S, Garmin, Kansas, USA). In order to prevent cross-contamination of *B. pseudomallei* between holes, the ‘sandak’ and the hand trowel were cleaned with water before sterilizing with 70% ethanol between holes. Then,

all sealed bags of soil samples were transported in light-proof bag back to the laboratory for analysis.

3.2.2 Ashdown's selective broth preparation

15 g of tryptone (Oxoid, Thermo Fisher Scientific Co., MA, USA) was mixed together with 5 ml 0.1 % crystal violet (Sigma, St. Louis, MO, USA) and 1 L of UHQ water. The mixtures were autoclaved at 121 °C for 15 minutes after preparation. Then, it was allowed to cool to 55 °C before colistin (Sigma, St. Louis, MO, USA) was added aseptically to a final concentration of 50 mg/L.

3.2.3 Ashdown's selective agar preparation

24 g of tryptone, 64 ml of glycerol, 1% aqueous neutral red (Sigma, St. Louis, MO, USA), 8 ml of 0.1% crystal violet, 24 g of agar powder (Sigma, St. Louis, MO, USA) and 1600 ml UHQ water have been mixed and autoclaved. Then it were allowed to cool to 55 °C. Gentamicin (Sigma, St. Louis, MO, USA) was added into the agar to a final concentration of 50 mg/L. Then, the agar was poured onto Petri dished and allowed to cool before storing at 4 °C. The plastic of Petri dish were labeled accordingly indicating the concentration of the antibiotics.

3.2.4 Soil sample culture

During soil sample culture, firstly, 20 g of soil was added into 50 ml tube containing 20 ml of deionized water. Then, the mixture was incubated at 37 °C shaking at 240 rpm for up to 39 hours. After incubation, 100 µl of the supernatant were inoculated onto Ashdown's agar and incubated at 37 °C. Another 10 ml of the supernatant also was inoculated into 30 ml of Ashdown's broth and incubated at 37 °C. After the second and seventh day's post-inoculation,

10 μ l loopful of surface liquid culture was streaked onto Ashdown's agar respectively. Colonies with morphologies that resemble that of *Burkholderia* spp. were subculture onto fresh Ashdown's agar to get pure cultures. Figure 3.2.4 below illustrates the soil sample culture method.

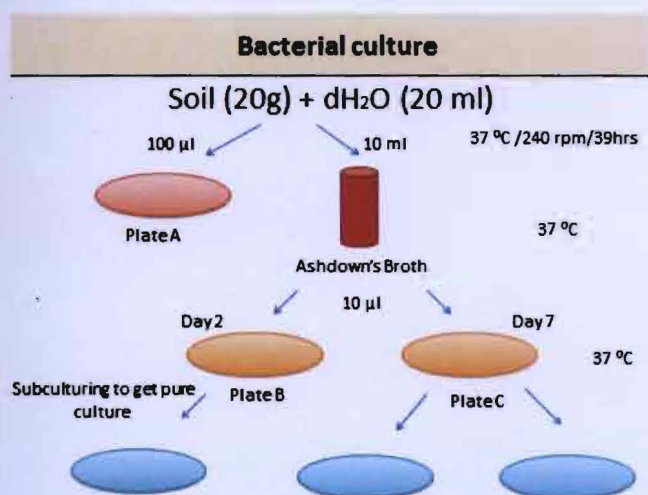


Figure 3.2.4: Bacterial culture method.

3.2.5 Chromosomal DNA extraction

DNA extraction were done on bacterial isolates by suspending bacterial colonies with a sterile solution containing 10% Chelex 100 (Bio-Rad, CA, USA). The suspension was boiled for 10 minutes. Then, the final supernatant were recovered after centrifugation at 10 000 rpm for 10 minutes and stored at -20 °C.